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(54) Monoclonal antibody specific to human melanoma

(57) A monoclonal antibody M2590 which is specific to human melanoma may be reacted with a tissue or cell sample suspected of containing melanoma cells, and the extent of the antibody/antigen reaction measured.

GB 2 133 543 A

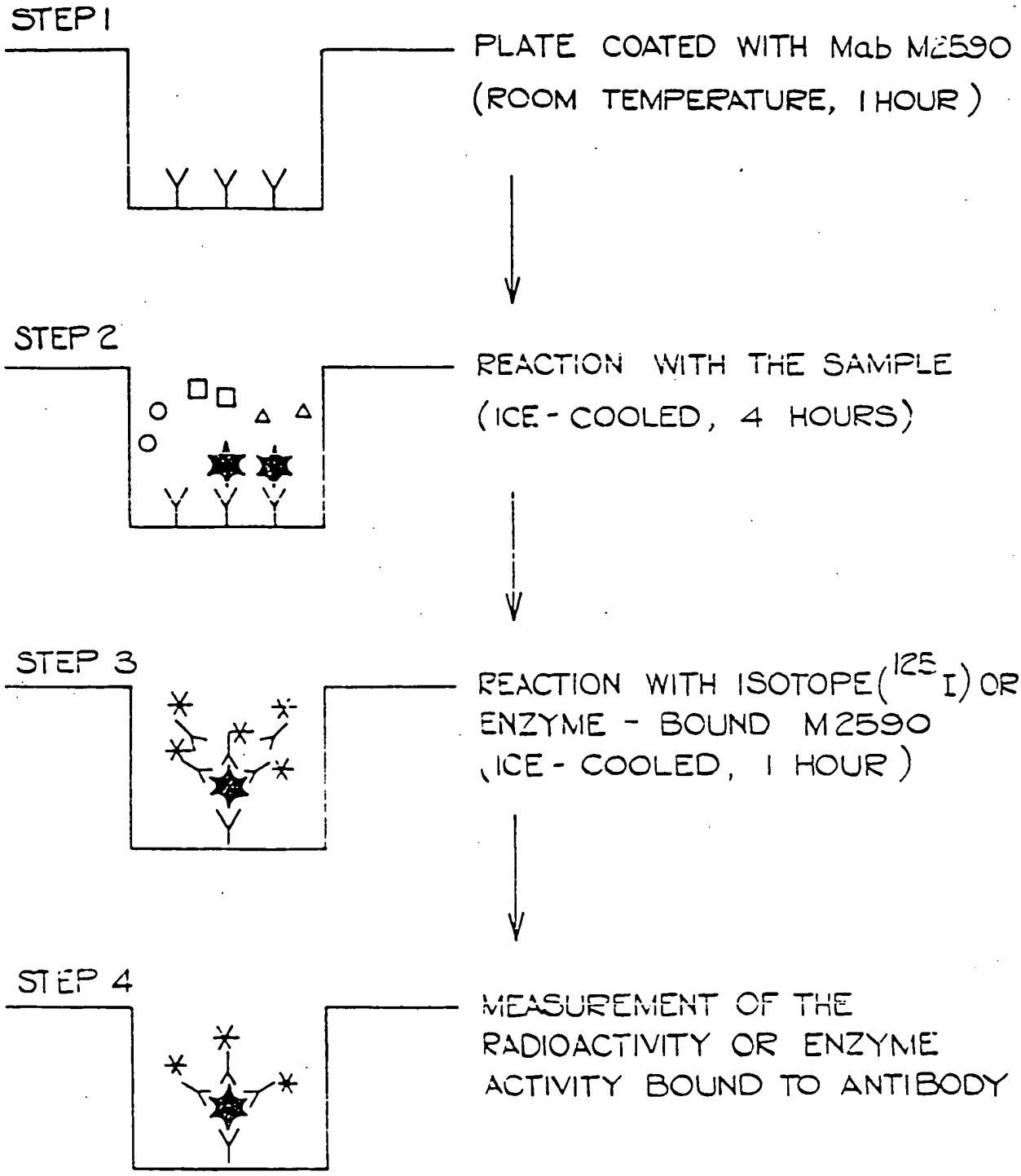

STEP 1
PLATE COATED WITH Mab M2590
(ROOM TEMPERATURE, 1 HOUR)
STEP 2
REACTION WITH THE SAMPLE
(ICE-COOLED, 4 HOURS)
STEP 3
REACTION WITH ISOTOPE (^{125}I) OR
ENZYME-BOUND M2590
(ICE-COOLED, 1 HOUR)
STEP 4
MEASUREMENT OF THE
RADIOACTIVITY OR ENZYME
ACTIVITY BOUND TO ANTIBODY

FIG. 1.

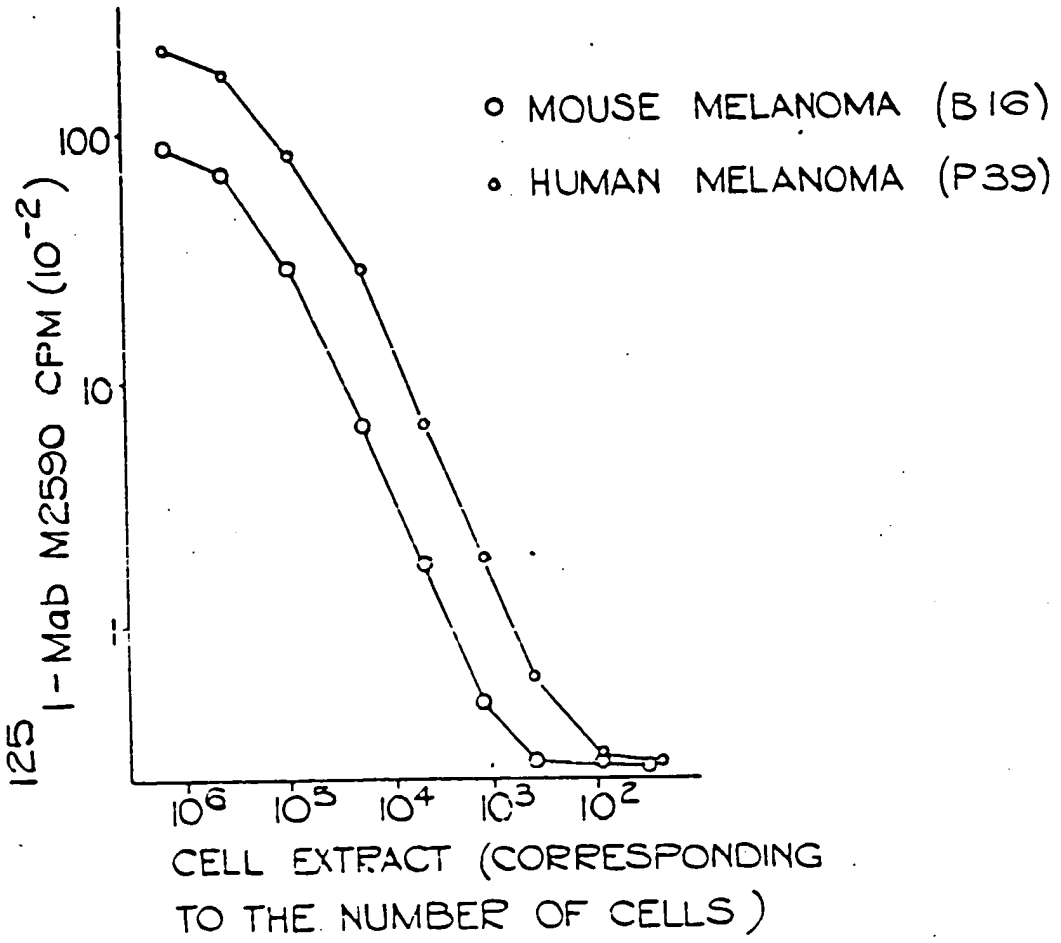
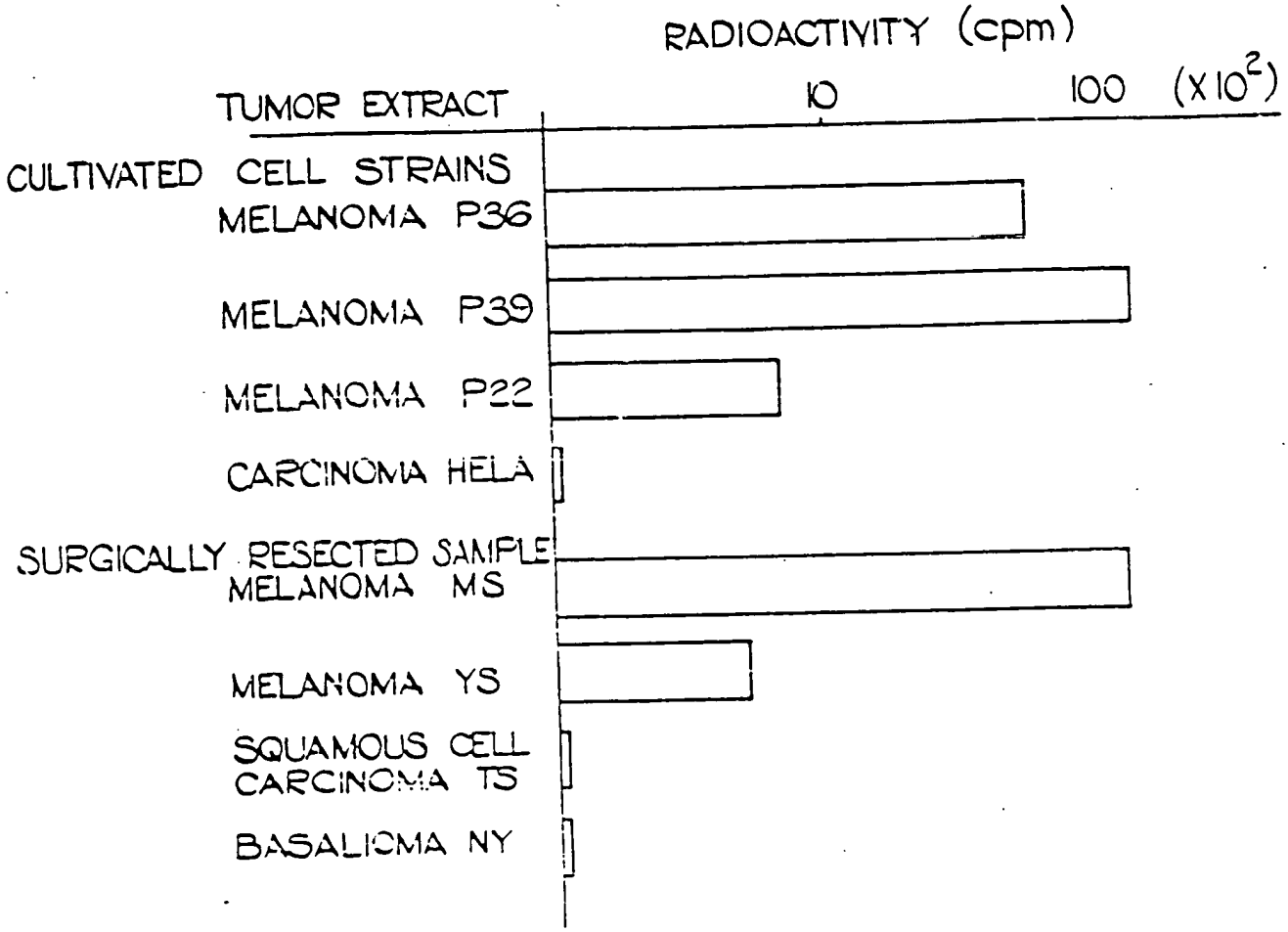

o MOUSE MELANOMA (B16)
o HUMAN MELANOMA (P39)
125 I-Mab M2590 CPM (10^{-2})
100
10
10^6 10^5 10^4 10^3 10^2
CELL EXTRACT (CORRESPONDING TO THE NUMBER OF CELLS)

FIG.2.


RADIOACTIVITY (cpm)
10
100
(X10²)
TUMOR EXTRACT
CULTIVATED CELL STRAINS
MELANOMA P36
MELANOMA P39
MELANOMA P22
CARCINOMA HELA
SURGICALLY RESECTED SAMPLE
MELANOMA MS
MELANOMA YS
SQUAMOUS CELL CARCINOMA TS
BASALIOMA NY

SPECIFICATION

Monoclonal antibody and methods for its use

BACKGROUND OF THE INVENTION

This invention relates to a reagent for use in diagnosing melanoma, said reagent comprising a monoclonal anti-melanoma antibody capable of recognizing a melanoma antigen common to mammals. The present invention also relates to diagnostic procedures for detecting melanoma employing the aforementioned reagent.

The present inventors have found on a T cell level the existence of a melanoma specific antigen common to different animal species expressed on the surface of melanoma cells (*Nature, 294*, 748–750, 1981).

SUMMARY OF THE INVENTION

An object of the present invention is to provide a diagnostic reagent comprising a monoclonal anti-melanoma antibody.

A further object of the present invention is to provide a monoclonal anti-melanoma antibody that is not species specific.

Another object of the present invention is to provide diagnostic kits including as one reagent therein a monoclonal anti-melanoma antibody.

Still another object of the present invention is to provide diagnostic procedures for detecting melanoma in mammals, particularly humans, employing a non-species dependent monoclonal anti-melanoma antibody.

Other objects of the invention will be apparent to the skilled artisan from the Detailed Description of the Invention hereinafter.

According to this invention, there is provided (1) a reagent for use in diagnosing human melanoma comprising a monclonal antibody capable of recognizing a melanoma antigen common to mammals, (2) diagnostic kits including said reagent and (3) immunoassays for detecting human melanoma antigens. The reagent can comprise the monclonal antibody and a moiety in association therewith, such as a radioactive element or an enzyme, or another carrier moiety.

In a preferred embodiment of this invention, the melanoma antibody is M2590 antibody.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is an explanatory view schematically illustrating a procedure for diagnosing melanoma in accordance with this invention;

Figure 2 is a graph plotting the detection of melanoma antigens using a radioimmunoassay embodiment of the present invention; and

Figure 3 is a graph showing the specificity of the present invention in the detection of human melanoma antigens by radioimmunoassay.

DETAILED DESCRIPTION OF THE INVENTION

The previously found melanoma specific antigenicity common to various animal species is also recognized by an antibody obtained by isoimmunization; thus, the antigen has been shown to be a melanoma specific antigen. Specifically, melanoma cells derived from C57BL/6 mice are treated with mitomycin, and administered intraperitoneally to C57BL/6 mice a number of times for immunization. The spleen cells of the animals are fused with P3U1 myeloma cells using polyethylene glycol to produce a monoclonal antibody. This antibody is a mouse antibody of the IgM class, but in spite of its production by immunization with mouse melanoma, it reacts not only with mouse melanoma but also with melanomas from other animals such as human and hamster melanomas. However, the monoclonal antibody has been found to have such specificity that it does not react at all with neuroblastoma, myeloma, fibrosarcoma, squamous cell carcinoma, acanthoma, cervical carcinoma and lymphoma, or with normal tissues (from the skin, eyes, cerebrum, etc.) derived from humans and mice.

Biochemical analysis has shown that the human or mouse melanoma antigen is a glycoprotein having a molecular weight of 31,000. In particular, experiments involving treatment with exoglycosidase and treatment with tunicamycin have led to the discovery that the antigenicity recognized by the monoclonal anti-melanoma antibody obtained as in the Reference Example hereinbelow (termed "M2590 antibody") is asparagine-bound sugar chains having sialic acid at the terminals.

Generally, a monoclonal antibody recognizes only one antigenic determinant of antigen molecules. When a specified antigenic determinant is a protein, only one such determinant is present on one molecule. Hence, only one molecule of monoclonal antibody can be combined with one antigen molecule. However, the monoclonal antibody used in this invention recognizes a tumor antigen composed of sugar chains. This means that many sugar chains are present on one antigen molecule, and therefore many antibody molecules can combine with one molecule of antigen. Accordingly, the monoclonal antibody of this invention, with its reactivity of high specificity, would be expected to exhibit high sensitivity when employed as a diagnostic or therapeutic agent.

Since, as stated above, the monoclonal antibody used herein such as M2590 antibody can equivalently react with non-human animal melanoma cells and human melanoma cells, the results of experiments on animal models will be directly applicable to humans. In view of this, the aforesaid antibody is an ideal agent for diagnosing melanoma. In particular, an immunoassay involving the use of the

monoclonal antibody in combination with a label such as isotopes or enzymes is expected to be an excellent diagnostic tool.

The production of M259C antibody as a preferred melanoma antibody used in this invention is shown by the following Reference Example.

Reference Example

(1) Sensitization of mouse with antigen

In a culture fluid RPMI-1640 (containing 4% bovine fetus serum, made by Gibco Co.), 1×10^7/ml of mouse B-16 melanoma cells (*J. Natl. Cancer Inst., 32,* 535-545, 1964) were reacted with 100 μg/ml of mitomycin C at 37°C for 45 minutes. The B16-melanoma cells (1×10^6) treated with mitomycin C were administered intraperitoneally to C57BL/6 mice once a week, and this immunization was repeated over 10 weeks. Furthermore, three days before cell fusion, 1×10^6 B16-melanoma cells treated with mitomycin C were intravenously injected into the animals. Three or four days later, the spleens were taken out from the mice, and were broken into pieces to obtain a RPMI-1640 cell suspension.

(2) Hybridization

10 ml of an RPMI 1640 suspension containing 1×10^7 myeloma cells P3U1 (*Curr. Top. Microbiol. Immunoil., 81,* 1-7, 1978) and 10 ml of a suspension containing 1×10^8 spleen cells prepared in (1) above (if desired, both of them may be used in a number of 1×10^8; in other words, the mixing ratio of the myeloma cells to the spleen cells may be 1:10 to 1:1) were put in a 50 ml centrifugal tube, and centrifuged at room temperature for 10 minutes at 400 × g. The supernatant was completely removed, and the residue was put in a constant temperature vessel at 37°C. While the cells were slowly mixed with the tip of a pipette, 1 ml of heated 50% polyethylene glycol (PEG) solution was added slowly over the course of 1 minute. For another 1 minute, the suspension was stirred by the same pipette. Then, 2 ml of heated RPMI-1640 was added slowly over the course of 2 minutes with stirring. Additionally, 7 ml of RPMI-1640 was added over 2 to 3 minutes. The mixture was centrifuged at room temperature for 10 minutes at 400 × g. The supernatant was removed, and 10 ml of RPMI-1640 containing 10% fetal calf serum was added. The mixture was lightly stirred. The cell suspension was dividedly poured in an amount of 0.1 ml per well onto a flat-bottomed micro test plate having 96 wells, and cultivated in a carbon dioxide incubator.

(3) Screening

On the next day to the day of starting the cultivation, 0.1 ml of HAT (hypoxanthine, aminopterin, thymidine) culture fluid was added, and after the lapse of 2, 3, 5, 8 and 11 days, one half of the supernatant in each well was sucked, and 0.1 ml of a fresh supply of HAT culture fluid was added. Thereafter, the culture medium was exchanged with HT (hypoxanthine, thymidine) culture fluid every 3 or 4 days.

As target cells, (1) B16 mouse melanoma cells, (2) human melanoma cells and (3) EL-4 lymphoma cells derived from C57BL/6 mice were used. 5×10^5 of the target cells were reacted with 50 μl of the supernatant culture liquid of the hybridoma (including the antibody) at 0°C for 50 minutes. The reaction was carried out on a 96-well microtiter polystyrene plate (made by Dynatech Lab. Co.). The cells were then washed by centrifugation, and reacted with 50 μl of antimouse Ig antibody (50,000 cpm/well) labelled with ^{125}I at 0°C for 1 hour. The reaction product was well washed, and dried, and then its radioactivity was measured by a gamma counter (made by Dynaboard Co.).

That antibody which reacted with (1) but not with the target cells (2) and (3) was provisionally regarded as a mouse melanoma specific antibody (M562 antibody), and the next step was performed. That antibody which reacted with (1) and (2) but not with (3) was dealth with as an antibody (M2590 antibody) recognizing a melanoma antigen common to different species. Those antibodies which reacted with all of the target cells (1), (2) and (3) or did not react with any of the target cells were excluded as non-specific antibodies.

(4) Cloning of cells capable of producing the desired antibody

Limiting dilution was used. When by screening, a well was found in which hydridoma cells producing a specific antibody existed, cloning was performed on that well by the limiting dilution method. In performing cloning, the cells were adjusted so that their concentration became 30 cells/20 ml. The cell suspension was sown in an amount of 0.2 ml portions onto wells of a 96-well flat-bottomed microtiter plate. Five days after cloning, 0.1 ml of HA (hypoxanthine, aminopterin) culture fluid was added to each well, and 12 days later, one-half of the culture medium was exchanged with fresh HA culture fluid. Thereafter, the culture medium was exchanged every 3 or 4 days with fresh HA culture fluid.

Cells producing M 562 antibody were designated as D_2, and cells producing M2590 antibody, as D_{10}.

(5) Purification of antibody

1×10^8 hybridoma cells were administered intraperitoneally to each of (BALB/C × C57BL/6) F_1 mice, and the ascites containing the antibody was collected after 10 days. Ten milliliters of the ascites was centrifuged, put into a dialysis tube, and dialyzed against 0.001M phosphate buffer (pH 6.5) for 40 hours. By this operation, all of the monoclonal antibodies could be precipitated. The precipitated monoclonal antibody was dissolved in a

small amount of 3% NaCl solution, and dialyzed against 0.1 M phosphate/sodium chloride buffer (pH 7.2). As a result, most of the other proteins could be removed together with the supernatant. By repeating the above procedure 2 to 3 times, the monoclonal antibody having a purity of more than 95% could be obtained. The immunoglobulin classes of the M2590 and M562 antibodies was IgM, and moreover, they were euglobulins.

The melanoma antibodies obtained as above can be used as a melanoma diagnostic agent, for example, in the form of a solution.

The following Example illustrates a method of diagnosing melanoma using the melanoma diagnostic agent of this invention.

Example

(1) Solid-phase immunoassay

A solution of purified M2590 antibody (purified by isoelectric precipitation) in a concentration of 2 mg/ml was prepared by using 0.01M phosphate/sodium salt buffer (pH 7.2). 60 μl of the antibody solution was added per well to a 96-well U-shaped polystyrene plate (made by Dynatech Lab. Co.), and reacted at room temperature for 1 hour. Then 0.5% bovine serum albumin (BSA) was added, and the plate was washed three times with phosphate/sodium chloride buffer (BSA-PBS) to remove the antibody not bound to the plate. Furthermore, 100 μl of BSA-PBS was added, and the reaction was performed at room temperature for 1 hour to close up protein-reaction groups (step 1). After thorough washing, 40 μl of a tumor antigen extract or a supernatant of a culture of cancer cells prepared as shown below was added per well, and reacted at 4°C for 4 hours (step 2). Four hours later, the plate was washed three times with STN buffer (pH 7.6) (containing 20 mM Tris, 0.15M sodium chloride, 0.2% sodium nitride, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 1% NP-40 surfactant), and then reacted with 50 μl (10,000 cpm) of M2590 antibody labeled with ^{125}I according to the method as described in *Biochemical Journal, 108*, 611-618. (1968) (step 3). After performing the reaction at 40°C for 1 hour, the reaction product was well washed, and its radio-activity was measured by a gamma counter (step 4). This operation is shown in Fig. 1.

The aforesaid tumor antigen extract and the supernatant of the culture of cancer cells were prepared as follows:

Tumor cells (corresponding to 2×10^7) extracted from tissues or fragments of tissues surgically resected and corresponding to 100 mg in weight were reacted with 1 ml of STN buffer (Iph—isoelectric pH 7.6) at 4°C for 1 hour. The reaction mixture was centrifuged at 10,000 rpm for 5 minutes to obtain a supernatant. A cancer antigen was extracted and present in the supernatant.

Cancer cells (2×10^6) were cultivated in RPMI-1640 containing 4% calf serum, and the supernatant of the culture broth was obtained.

(2) Preparation of calibration curve and the accuracy of the solid-phase immunoassay:

Tumor antigens were produced from varying numbers of C57BL/6 melanoma (B16) cells or human melanoma cells (P-39) by the procedures described in the last two paragraphs of section (1) above. The resulting tumor cell extracts of varying antigen concentrations were added to the immunoassay system described in (1) above and examined. The result is that, as shown in Fig. 2, when an extract corresponding to 10^6/ml of tumor cells was sequentially diluted to a ratio of 1:10, the detection showed a nearly linear fall at a concentration of 10^5/ml and over, and the minimum detection limit was 10^3-10^2/ml.

This experimental result shows that the solid-phase immunoassay using this antibody (M2590) is very accurate and permits detection of a very small amount of a melanoma antigen.

(3) Specificity of the solid-phase immunoassay

To examine the specificity of the solid-phase immunoassay method, tumor antigen extracts were prepared in accordance with the method described in section (1) above from three kinds of human melanoma cells (P-36, P-39, P-22), hamster melanoma cells, two kinds of mouse melanoma cells (B-16, S91), human cervical carcinoma (Hela), surgically resected fragments from a human melanoma patient (two cases), human skin cancer (squamous cell carcinoma) and resected samples of basalioma. These tumor antigen extracts were subject to solid-phase immunoassay by the method described in section (1) above. The result was that as shown in Fig. 3, extracts of melanoma cells derived from humans, hamsters and mice, whether they were cultivated cell strains or surgically resected samples, could be detected by immunoassay, but the antibody did not at all react with cancer cells other than melanoma cells, such as human cervical carcinoma, squamous cell carcinoma and acanthoma. The results are shown in Fig. 3.

The following conclusions can be drawn from the above results.

(1) Human melanoma can be specifically diagnosed by using a cell or tissue extract.

(2) Immunoassay using the monoclonal anti-melanoma antibody such as M2590 antibody has high sensitivity, and can detect a melanoma antigen in a human melanoma cell extract in which 1000 to 500 melanoma cells exist.

While the invention has been described in detail and with reference to specific embodi-

ments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope thereof. For example, different immunoassay testing procedures, for example where the monoclonal antibody, (labeled or unlabeled) is attached to other conventional solid-phase supports such as glass beads or where other labels such as an enzyme, a fluorescent compound, etc. are used could be employed, or an indirect method using secondary antibody. Thus, the monoclonal antibody could be attached to various conventional carrier moieties or materials such as a test tube, a carrier bead, a particulate polymer, and the like.

CLAIMS

1. A reagent for use in diagnosing human melanoma, said reagent comprising a monoclonal antibody capable of recognizing a melanoma antigen common to mammals and a label or carrier moiety or material associated with said antibody.

2. The reagent of Claim 1 wherein the monoclonal antibody is M2590 antibody.

3. As a component of a diagnostic reagent kit for detecting human melanoma, a solid phase having attached thereto a monoclonal anti-melanoma antibody capable of recognizing a melanoma antigen common to mammals.

4. The component of Claim 3 wherein the solid phase comprises a support in or on which an immunoassay can be carried out.

5. The component of Claim 3 wherein the monoclonal antibody is M2590 antibody.

6. The component of Claim 4 wherein the monoclonal antibody is M2590 antibody.

7. The reagent of Claim 1 wherein said label moiety is a label moiety used in immunoassays.

8. The reagent of Claim 7 wherein the label moiety is a radioactive element.

9. The reagent of Claim 8 wherein the label moiety is an enzyme.

10. A procedure for detecting the presence of melanoma in a tissue or cell sample, said procedure comprising incubating a monoclonal anti-melanoma antibody capable of recognizing a melanoma antigen common to mammals with an extract of tissue or cells suspected of containing melanoma cells and determining the amount of said antibody which reacts with any antigen present in said extract.

11. The procedure of Claim 10 wherein the monoclonal antibody is M2590 antibody.

12. The procedure of Claim 11 wherein at least a portion of the monoclonal antibody used carries a label enabling measurement of the amount of labeled antibody which reacts with any antigen present in the extract.

13. The procedure of Claim 13 wherein the label is a radioactive isotope.

14. The procedure of Claim 13 wherein the label is an enzyme.

15. The procedure of Claim 10 wherein a sandwich assay is employed, with unlabeled monoclonal anti-melanoma antibody first reacting with only melanoma antigen present in the extract and then labeled anti-melanoma antibody reacting with said antigen, the amount of labeled antibody so reacting being measured.

16. A reagent kit for detecting human melanoma comprising a monclonal antibody capable of recognizing a melanoma antigen common to mammals and a substrate for carrying out an immunoassay.

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